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Mycorrhizal dynamics under elevated CO₂ and nitrogen fertilization in a warm temperate forest

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Abstract We examined the response of mycorrhizal fungi to free-air CO₂ enrichment (FACE) and nitrogen (N) fertilization in a warm temperate forest to better understand potential influences over plant nutrient uptake and soil carbon (C) storage. In particular, we hypothesized that mycorrhizal fungi and glomalin would become more prevalent under elevated CO₂ but decrease under N fertilization. In addition, we predicted that N fertilization would mitigate any positive effects of elevated CO₂ on mycorrhizal abundance. Overall, we observed a 14% increase in ectomycorrhizal (ECM) root colonization under CO₂ enrichment, which implies that elevated CO₂ results in greater C investments in these fungi. Arbuscular mycorrhizal (AM) hyphal length and glomalin stocks did not respond substantially to CO₂ enrichment, and effects of CO₂ on AM root colonization varied by date. Nitrogen effects on AM fungi were not consistent with our hypothesis, as we found an increase in AM colonization under N fertilization. Lastly, neither glomalin concentrations nor ECM colonization responded significantly to N fertilization or to an N-by-CO₂ interaction. A longer duration of N

fertilization may be required to detect effects on these parameters.

Keywords Mycorrhizal fungi · Nitrogen fertilization · Free air CO₂ enrichment · Temperate forest · Glomalin

Introduction

Humans are augmenting concentrations of CO₂ in the atmosphere by burning fossil fuels, clearing forests, and converting land to intensive agricultural systems (Schimel et al. 1995). In fact, the amount of CO₂ in the atmosphere is rising by approximately 3 Pg carbon (C) per year (Keeling et al. 1995). Atmospheric enrichment of CO₂ tends to enhance net primary production (NPP) and biomass of land plants (DeLucia et al. 1999; Wang 2007) which could lead to sequestration of anthropogenic C emissions in terrestrial pools (van Veen et al. 1991; Gorissen 1996; Ineson et al. 1996). Microbial symbionts such as mycorrhizal fungi can contribute to C sequestration by increasing nutrient uptake by plants to sustain higher NPP levels. In addition, arbuscular mycorrhizal (AM) fungi produce glomalin, a recalcitrant glycoprotein that remains in the soil even after the fungus has senesced (Wright and Upadhyaya 1996). This compound could represent a globally significant pool of C that might be altered under global change (Treseder and Turner 2007).

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Plants rely upon mycorrhizal fungi to acquire nutrients such as phosphorus and nitrogen (N) for growth (Smith and Read 1997). Mycorrhizal fungi form symbiotic networks with plant roots. The fungi scavenge nutrients from soils and transfer these nutrients to the host plant in exchange for carbohydrates. The growth and abundance of mycorrhizal fungi should increase when rates of photosynthesis or nutrient demand increase (Norby et al. 1987; Diaz 1996; Hodge 1996) such as when CO₂ levels are high. Indeed, CO₂ enrichment consistently increases mycorrhizal abundance in field studies (Treseder 2004). However, most of these measurements have been conducted in agricultural areas and temperate grasslands. By contrast, few studies have focused on the response of mycorrhizal fungi to elevated CO₂ in temperate forests (but see Rey et al. 1997; Kasurinen et al. 1999). This consideration is important because temperate forests, together with temperate grasslands, are estimated to comprise a substantial sink for atmospheric CO₂ (Chapin et al. 2002).

Mycorrhizal fungi should be particularly important for augmenting plant nutrient uptake under elevated CO₂ in ecosystems where soil nutrients are limiting. Temperate forests tend to be N-limited, meaning that NPP consistently increases under N fertilization (Vitousek and Howarth 1991). In contrast, mycorrhizal abundance declines by 15% on average following N additions (Treseder 2004). However, few studies have examined interactions between elevated CO₂ and N fertilization, especially under field conditions. Nitrogen fertilization can negate the effect of elevated CO₂ on mycorrhizal fungi (Klironomos et al. 1997; Rillig and Allen 1998), although in some cases no interaction is observed (Tingey et al. 1995; Walker et al. 1997; Lussenhop et al. 1998).

In this study, we examined the dynamics of ECM fungi and AM fungi in soils of a loblolly pine plantation exposed to free-air CO₂ enrichment and N fertilization. Plant responses to elevated CO₂ are limited by N availability in this ecosystem (Oren et al. 2001; Finzi et al. 2002) and this response may be strengthening over time (Finzi et al. 2006a, b). We tested three hypotheses regarding the abundance of mycorrhizal fungi under elevated CO₂ and N fertilization. First, we hypothesized that mycorrhizal abundance and glomalin stocks would increase under elevated CO₂ as transfer of photosynthate to

mycorrhizal roots increases. Second, we predicted that mycorrhizal abundance and glomalin would decline under N fertilization, because plants would reduce investment in nutrient acquisition from soils under these conditions. Lastly, we expected that N fertilization would mitigate the positive effects of elevated CO₂ on mycorrhizal abundance and glomalin.

Materials and methods

Site description

This study was conducted at the FACE experiment in the Duke Forest (Orange County, North Carolina, USA). This experiment consists of twelve 30 m diameter plots: a “prototype” CO₂-enriched ring and its paired reference ring, both established in June 1994; three additional CO₂-enriched rings plus three paired control rings, each established in August 1996; and four control rings established in January 2005. The CO₂-enriched plots were maintained at 200 $\mu\text{mol mol}^{-1}$ above ambient CO₂ concentration, except when air temperature fell below 4°C. Added CO₂ is delivered throughout the height of the canopy, from near ground-level to tree top (J. Pippen, personal communication). In January 2005, each ring was divided into four equal sectors, and N fertilization (10 g N m⁻² year⁻¹) commenced in two randomly chosen sectors in each ring. Ion exchange resin bags indicate a large increase in soil N availability in the fertilized plots (A. Finzi, personal communication). The forest is a 23-year-old loblolly pine (*Pinus taeda* L.) plantation, in which trees are arrayed in a 2.4 × 2.4 m grid (for additional site details, see Finzi and Schlesinger 2003). Loblolly pine is ectomycorrhizal. Understory species include perennial vines and shrubs and seedlings and saplings of the tree species *Acer rubrum* L., *Carya glabra* Mill., *Cercis canadensis* L., and *Liquidambar styraciflua* L. (Springer and Thomas 2007). Many of these plants are arbuscular mycorrhizal (Constable et al. 2001; St Clair and Lynch 2005; Thiet and Boerner 2007).

Soil sampling occurred in May 2005, July 2005, November 2005, Feb 2006, May 2006, and August 2006. We measured AM colonization, ECM colonization, and/or glomalin in these samples. Within each soil sector of each ring, two 10 cm diameter × 10 cm

deep cores were collected from random locations and compiled into one bag. Altogether, there were a total of 48 samples. The samples were placed into a cooler with ice packs and shipped overnight to UCI, where they were stored at -20°C .

ECM colonization

We measured percent root length colonized by ECM fungi on fine (<2 mm diameter) roots collected on May 2005, November 2005, May 2006, and August 2006. Roots were isolated by hand sieving, and approximately 60 cm of fine roots were randomly selected from each sample. The roots were rinsed three times with deionized water and then examined for ECM tissues at $40\times$ magnification (Olympus Sz40, Olympus Inc., Melville, NY, USA). We used a point intersection method to estimate the percentage of root length covered by ECM sheaths (Brundett et al. 1996).

AM colonization

AM colonization was assessed on all sampling dates. Approximately five 1 cm-lengths of fine roots were selected at random from each sample for each collection date. Roots were stained with Trypan Blue as detailed in Koske and Gemma (1989), and mounted with PVLG medium onto slides (Koske and Tessier 1983). We used the magnified intersections method (McGonigle et al. 1990) and a Nikon phase-contrast microscope (Nikon Eclipse e400, AG Heinze, Lake Forest CA, USA) to quantify the presence of arbuscules, vesicles, or intraradical hyphae. One hundred intersections or more were assessed for each sample, from which we calculated percent root length colonized.

AM hyphal length

For each sampling date, we measured AM hyphal length by extracting hyphae from 10 g of air-dried soil per sample as detailed in Treseder et al. (2007). Briefly, we homogenized the soil in a detergent mixture, used sieving to isolate the hyphae, and then collected the hyphae on a polycarbonate filter. We examined the filter for the presence of hyphae by using a phase contrast microscope at $200\times$ magnification. Hyphal lengths were estimated with the point-

intersect method (Paul and Clark 1996); 100 points were scanned per sample. Hyphae from AM fungi were distinguished from those of non-AMF based on morphological features such as irregular walls, lack of septa, and angular branching (Bonfante-Fasolo 1986).

Glomalin

To measure concentrations of glomalin, we used an enzyme-linked immunosorbent assay (ELISA; Wright and Upadhyaya 1996; Wright et al. 2000). Glomalin was isolated from 1 g of soil by adding 8 ml of 50 mM sodium citrate (pH 8.0). The soil was autoclaved at 121°C for 1 h, followed by centrifuging at 5000 g for 15 min. The supernatant was retained and stored at 4°C . Glomalin concentrations in the extracts were assayed with an ELISA procedure described in Wright and Upadhyaya (1996). In short, the extract was dried overnight in microtiter plate wells and then incubated with a glomalin-specific monoclonal antibody (MAb32B11). We determined glomalin concentrations (as mg glomalin g^{-1} dry soil) by using a microplate reader equipped with a 405 nm filter (EI800, Bio Tek Instruments, Winooski, VT, USA). The values were compared with those of a glomalin standard derived from fresh AM hyphae. Our glomalin concentrations represent easily-extractable immunoreactive soil protein (EE-IRSP), following nomenclature proposed by Rosier et al. (2006). Glomalin was measured in May 2005 and May 2006.

Statistics

To test our hypotheses, we conducted a series of split-split plot analyses of variance (ANOVA; Sokal and Rohlf 1995). Dependent variables were ECM colonization, AM colonization, AM hyphal lengths, or glomalin. Independent variables were CO_2 treatment, N fertilization, and sampling date. The main factor was CO_2 , the split factor was N fertilization, and the sub-split factor was sampling date. In each ANOVA, the unit of replication was the plot (i.e., ring). Differences were considered significant when $P < 0.05$ and marginally significant when $P < 0.10$. Analyses were performed on ranked data, because we were unable to transform data to meet assumptions of normality and homogeneity of variances. Systat 10

software was used to conduct all analyses (SPSS, Chicago IL).

Results

ECM colonization

Atmospheric CO₂ enrichment was associated with a significant increase in ECM colonization (Fig. 1a, Table 1), but N effects were not significant for this functional group. There was also no significant CO₂ by N interaction. Although sampling dates varied significantly from one another, there were no signif-

icant interactions between sampling date and CO₂, N fertilization, or CO₂ × N fertilization.

AM colonization

Arbuscular mycorrhizal colonization was significantly greater in sections that were N fertilized versus those that were not fertilized (Fig. 1b, Table 1). Overall, this variable did not significantly differ between ambient and enriched CO₂. Nevertheless, a significant date by CO₂ interaction indicated that effects of CO₂ varied over time. No discernable seasonal or progressional pattern was apparent, though. In addition, we observed a marginally significant interaction between

Fig. 1 Abundance of ectomycorrhizal (a) and arbuscular mycorrhizal (b) fungi on roots under CO₂ and N enrichment in the Duke Forest. Average values across sampling dates are presented in right-most panel. Root colonization by ECM fungi was greater under elevated CO₂ compared to ambient CO₂ (Table 1). For AM fungi, N fertilization was associated with an increase in colonization level. Effects of elevated CO₂ varied over time for AM colonization. Error bars represent +1SE of three to four plots (elevated CO₂ treatment) or five to eight plots (ambient CO₂ treatment)

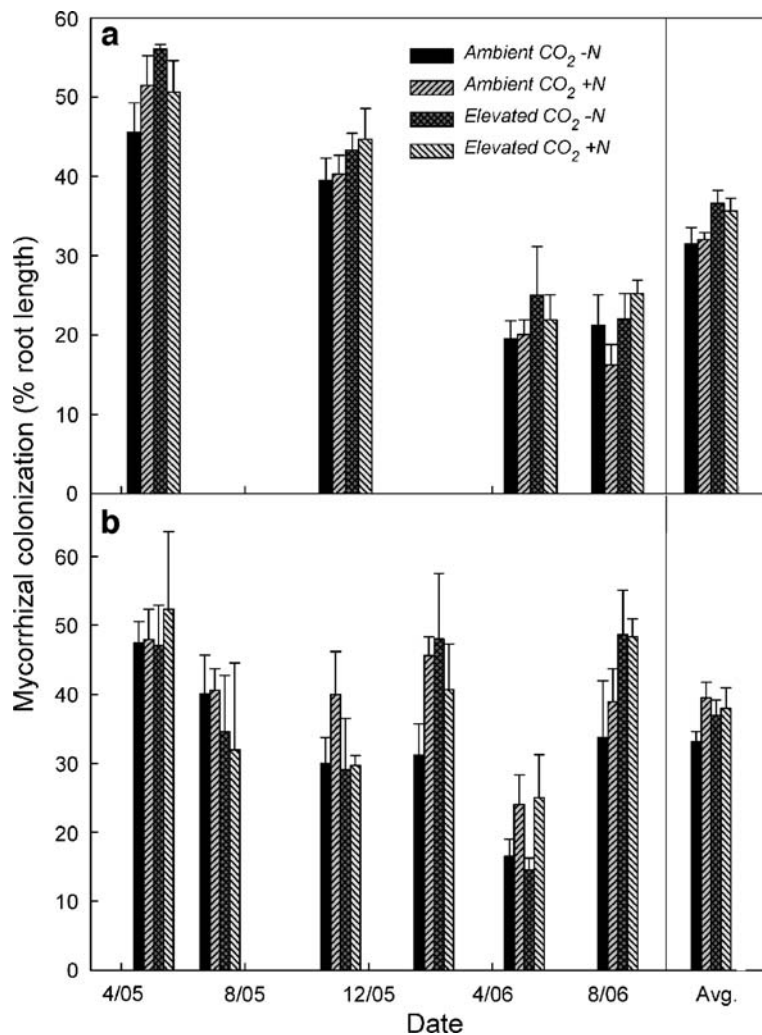


Table 1 ANOVA statistics for mycorrhizal responses to elevated CO₂, N fertilization, and sampling date

Response variable	Source	F ratio	Degrees of freedom	P value ^a
ECM colonization	Elevated CO ₂	6.403	1, 10	0.030
	N fertilization	0.009	1, 10	0.926
	Sampling date	68.658	3, 30	<0.001
	CO ₂ *N	0.029	1, 10	0.869
	CO ₂ *date	0.027	3, 30	0.994
	N × date	0.026	3, 30	0.994
	CO ₂ × N × date	0.943	3, 30	0.432
AM colonization	Elevated CO ₂	0.067	1, 6	0.804
	N fertilization	8.113	1, 6	0.029
	Sampling date	19.873	5, 30	<0.001
	CO ₂ × N	5.814	1, 6	0.052
	CO ₂ × date	2.754	5, 30	0.037
	N × date	0.584	5, 30	0.712
	CO ₂ × N × date	1.107	5, 30	0.377
AM hyphae	Elevated CO ₂	0.084	1, 10	0.778
	N fertilization	0.988	1, 10	0.344
	Sampling date	156.893	4, 40	<0.001
	CO ₂ × N	1.970	1, 10	0.191
	CO ₂ × date	2.172	4, 40	0.087
	N × date	0.753	4, 40	0.562
	CO ₂ × N × date	0.813	4, 40	0.524
Glomalin concentration	Elevated CO ₂	0.778	1, 10	0.398
	N fertilization	2.575	1, 10	0.140
	Sampling date	1.528	1, 10	0.245
	CO ₂ × N	1.940	1, 10	0.194
	CO ₂ × date	16.979	1, 10	0.002
	N × date	0.520	1, 10	0.487
	CO ₂ × N × date	0.992	1, 10	0.343

^a Significant *P* values in bold

CO₂ and N whereby N fertilization tended to increase AM colonization more under ambient CO₂ than under enriched CO₂. Colonization by AM fungi varied significantly over time, but we observed no significant interaction between date and N fertilization or between date, N fertilization, and CO₂.

AM hyphal length

Standing hyphal lengths of AM fungi did not respond significantly to CO₂, N fertilization, or their interaction (Table 1). Across sampling times, AM hyphal lengths averaged 0.44 ± 0.10 m g⁻¹ soil (mean \pm 1SE) in the ambient CO₂/ unfertilized treatment; 0.47 ± 0.07 m g⁻¹, ambient CO₂/N fertilized; 0.59 ± 0.15 m g⁻¹, elevated CO₂/ unfertilized; and 0.49 ± 0.18 m g⁻¹, elevated CO₂/N fertilized. Although values varied significantly by sampling date, there were no significant interactions between date and CO₂, N fertilization, or CO₂ × N.

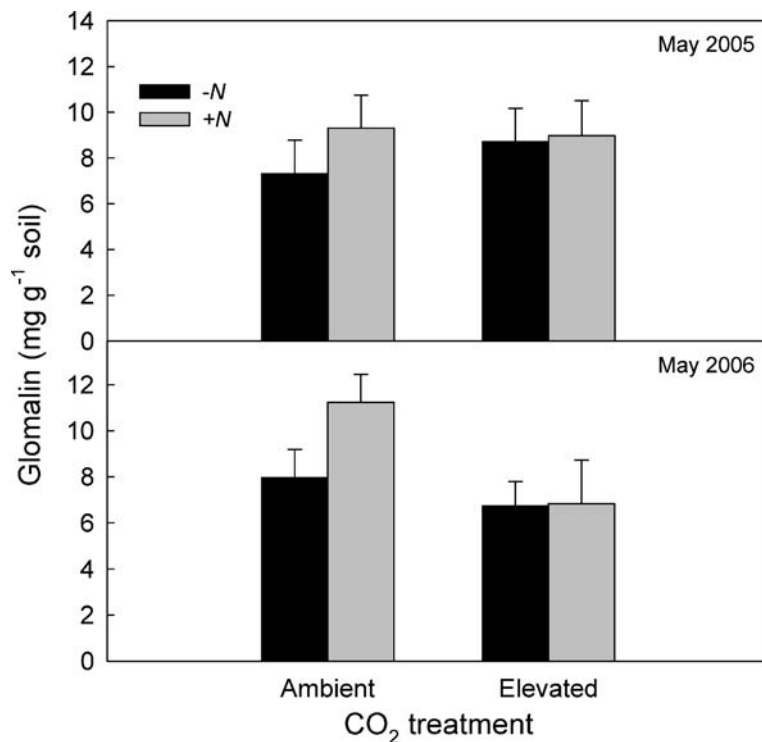
Glomalin

Overall, elevated CO₂ did not significantly affect glomalin concentrations (Fig. 2, Table 1). However, we observed a significant CO₂ by sampling date interaction. Compared to the ambient CO₂ treatment, glomalin concentrations in the elevated CO₂ treatment tended to be 6% higher in May 2005 but 29% lower in May 2006. There were no significant effects of N fertilization. In addition, there was no overall significant difference between the sampling dates. Likewise, we found no significant interactions for CO₂ × N fertilization, sampling date × N fertilization, or sampling date × CO₂ × N fertilization.

Discussion

We found that the proportion of root length colonized by ECM fungi increased in response to elevated CO₂

Fig. 2 Concentrations of glomalin (EE-IRSP) in soil in May 2005 and May 2006. Effects of elevated CO₂ varied significantly by sampling date (Table 1). Error bars represent +1SE of four plots (elevated CO₂ treatment) or eight plots (ambient CO₂ treatment)



in this warm temperate forest. Our results are consistent with those of Pritchard et al. (*in press*), who used minirhizotron imagery to document a marginally significant increase in production of ECM root tips in this site. In addition, investment in fine roots (indicated by annual increment of fine root biomass) is about 87% greater under elevated CO₂ (Matamala and Schlesinger 2000). Total standing stocks of ECM root tips should have increased accordingly, since fine root biomass and the proportion of fine roots colonized by ECM fungi were both augmented under CO₂ enrichment. These results support our first hypothesis that elevated CO₂ would result in greater C investments in ECM root tips to foster N uptake from soils. In the Duke FACE site, soil CO₂ respiration increases under elevated CO₂ (Andrews and Schlesinger 2001; Bernhardt et al. 2006), which may be partly attributable to an increase in C allocation to ECM fungi (Allen et al. 2000). We note that a recent study by Parrent and Vilgalys (2007) found no significant increase in ECM hyphae under elevated CO₂. The increase in investment in ECM fungi may have been allocated preferentially to root-associated structures rather than extramatrical hyphae.

The dominant tree species, loblolly pine, appears to be enhancing its capacity for N uptake via multiple mechanisms. Net primary productivity increases in the Duke FACE site under CO₂ enrichment (e.g., DeLucia et al. 1999), and the increase in ECM colonization may be one factor that maintains this plant response (Finzi et al. 2006a). These supplementations of plant N uptake may be particularly important given that neither N mineralization nor the activity of N-targeting extracellular enzymes changes under CO₂ fumigation (Finzi and Schlesinger 2003; Finzi et al. 2006a). Here, ECM fungi may allow plants to directly acquire organic N compounds without relying upon transformations by decomposers (Read et al. 2004).

The community composition of ECM fungi has not shifted appreciably under CO₂ enrichment in the Duke FACE experiment (Parrent et al. 2006), indicating that ECM species in this site do not differ substantially from one another in the degree to which they proliferated under CO₂ fumigation. In non-N-fertilized areas, ECM diversity is negatively correlated with net N mineralization rates (Parrent et al. 2006). We did not characterize the community

composition of ECM fungi in N-fertilized sections, but we observed no significant changes in abundance of ECM fungi under N fertilization. It is possible that different ECM species could have been supported by host plants under N fertilization, even though no overall shift in the standing biomass of ECM fungi occurred.

A recent meta-analysis reported that across biomes, mycorrhizal abundance increases by an average of 47% under CO₂ enrichment (Treseder 2004). Temperate forests tend to respond more weakly, averaging a 15% increase. The 14% increase in ectomycorrhizal colonization under elevated CO₂ observed in this study is therefore consistent with the previous temperate forest studies.

Arbuscular mycorrhizal colonization also responded to elevated CO₂, but the degree and direction of response varied over time. There were no apparent seasonal trends in CO₂ effects on AM fungi. Instead, it is possible that the phenology of individual species of AM host plants may have influenced AM colonization levels, since plant species in this forest vary in the degree to which they are CO₂-sensitive (Mohan et al. 2007). In field or greenhouse-based CO₂ experiments, AM hyphal lengths increase in about half the cases (Treseder and Allen 2000). The remaining studies report no significant change. Thus, the lack of CO₂ response by AM hyphae in the Duke Forest is not unprecedented. Because AM colonization did not consistently increase under elevated CO₂ in our study, and because AM hyphal lengths were not significantly altered, our hypothesis that mycorrhizal abundance would become more prevalent under elevated CO₂ was rejected with respect to AM fungi.

Also in contradiction to our first hypothesis, we observed little evidence for changes in glomalin stocks under elevated CO₂ aside from a date-by-CO₂ interaction. Carbon dioxide concentrations had been manipulated for at least nine years prior to the onset of sampling. In addition, radiocarbon signatures of purified easily extractable glomalin indicate residence times of seven years in this site (K.K. Treseder, unpublished data), which are comparable to those estimated in Hawaiian rainforests (7–42 years, Rillig et al. 2001). Because CO₂ manipulations occurred over a similar timescale as glomalin residence times, we expect that any CO₂ effects on glomalin stocks should have been detectable. The date-by-CO₂ interaction, though significant, was subtle and did not

coincide strongly with changes in AM colonization or hyphal length over time. Standing stocks of glomalin are influenced by decomposition rates as well as production rates, and interannual variability in soil moisture or temperature may have elicited differing responses by the community of decomposers that target glomalin. Few previous studies have measured immunoreactive glomalin under CO₂ enrichment, although Rillig et al. (1999) found that glomalin concentrations increased under elevated CO₂ in soil aggregates of 0.25–1 mm diameter in two grasslands. Moreover, glomalin concentrations are not always correlated with AM abundance in field studies (e.g., Lutgen et al. 2003; Rillig et al. 2003), potentially because glomalin production and decomposition may be influenced by different environmental variables (Treseder and Turner 2007).

The concentrations of EE-IRSP glomalin in the Duke Forest (8.2 mg g⁻¹ soil) were high compared with other published field measurements, which range between 0.001 mg g⁻¹ soil in a semiarid rangeland (Bird et al. 2002) to 5 mg g⁻¹ soil in Hawaiian rainforest (Rillig et al. 2001; Treseder and Turner 2007). Although loblolly pine is ECM, plants in the understory of the Duke Forest are predominantly AM. On average, 33 to 40% of root length in this ecosystem was colonized by AM fungi, depending on treatment. This average is within the range of those reported for AM-dominated ecosystems such as deserts (33%), temperate grasslands (36%), and tropical forests (36%, Treseder and Cross 2006). The large glomalin stocks that we observed indicate that this compound could constitute a substantial C pool within the soil in this warm temperate forest.

Our second hypothesis predicted that abundance of mycorrhizal fungi and glomalin should decline under N fertilization. However, we did not observe any significant decreases in ECM colonization, AM colonization, AM hyphal length, and glomalin concentration in response to N addition, so we reject this hypothesis. In fact, N fertilization was associated with a significant increase in AM colonization, which is inconsistent with many other studies (e.g., Bentivenga and Hetrick 1992; Ellis et al. 1992; Hutchinson et al. 1998; Grogan and Chapin 2000; Cornwell et al. 2001; Treseder and Vitousek 2001; Treseder 2004; van Diepen et al. 2007). The increase under N-fertilization may be related to the phosphorus status of plants, since phosphorus availability is relatively low in this

site (George et al. 2003). Arbuscular mycorrhizal fungi are thought to be especially important in facilitating plant uptake of phosphorus (Mosse 1973). If AM host plants were secondarily limited by phosphorus, they may have increased C investment in mycorrhizal fungi under N fertilization to alleviate an exacerbation of phosphorus limitation.

Although AM colonization was augmented by N-fertilization, we found that standing hyphal lengths of AM fungi did not respond accordingly. It is possible that AM community composition shifted under N enrichment, which has been reported in other field studies (Johnson et al. 1991; Klironomos et al. 1997; Eom et al. 1999; Egerton-Warburton and Allen 2000; Treseder and Allen 2002; Treseder 2005). In most of these experiments, *Glomus* species have become more abundant relative to other species. *Glomus* tends to produce fewer external hyphae per unit root length colonized (Klironomos et al. 1998; Dodd et al. 2000). If a similar response occurred in the Duke FACE experiment, then this mechanism may underlie the lack of a significant effect on AM hyphae.

Shifts in soil glomalin concentrations under N additions have been inconsistent in previous studies (Wuest et al. 2005; Treseder et al. 2007). In our field site, we found no significant N effect on glomalin stocks despite an increase in root colonization by AM fungi, which produce glomalin. Although glomalin concentrations tended to be higher under N fertilization in the ambient CO₂ treatment, this response was not significant. Nitrogen fertilization had been ongoing for only 0.3 to 1.6 years during the sampling period. Given the relatively long turnover time of glomalin, it is possible that a longer duration of N fertilization may be required to detect changes in glomalin concentrations.

Finally, our third hypothesis suggested that N fertilization should mitigate effects of elevated CO₂ on glomalin stocks and mycorrhizal abundance. However, we found no evidence of this particular interaction between CO₂ and N fertilization. Neither ECM colonization, AM hyphal length, or glomalin concentration displayed significant interactions between these two variables. We observed a marginally significant interaction between CO₂ and N for AM colonization, but not in the expected direction. Specifically, CO₂ fumigation reduced the positive effect of N fertilization on AM colonization. Thus, our results do not support the third hypothesis.

In conclusion, the abundance of ECM fungi increased under elevated CO₂, which may enable plants to enhance N uptake and maintain greater NPP. The increase in ECM fungi may contribute to C sequestration by increasing nutrient uptake by plants, which could in turn sustain higher NPP levels. The extent to which this mechanism will contribute to C sequestration may ultimately depend on decomposer responses to CO₂. The increase in ECM colonization is consistent with observations drawn from other temperate forests. In contrast, AM fungi and glomalin stocks did not respond consistently to elevated CO₂. Moreover, we found that AM colonization was significantly greater in areas that were N fertilized, suggesting that co- or secondary-limitation of plants by phosphorus may be a factor. Regarding changes in glomalin stocks, longer-term observations may be necessary to detect shifts in glomalin concentrations that could result from increased AM colonization under N fertilization. Additional changes in soil N content and plant growth as the N fertilization experiment proceeds could also have important delayed effects that are not yet evident. Overall, we suggest that any role played by mycorrhizal fungi in C sequestration in the system under alterations in CO₂ or N will likely be related to indirect influences on NPP and not necessarily due to direct changes in stocks of glomalin.

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